Electronic Supplementary Information

Interaction between GUVs and Catanionic Nanocontainers: a New insight into Spontaneous Membrane Fusion

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1. Materials

The lipids used to prepare giant unilamellar vesicles were: *L*-*α*-phosphatidylcholine (Egg, Chicken) (Egg-PC), *1*,*2*-dioleoyl-*sn*-glycero-*3*-phosphocholine (DOPC), *1*-palmitoyl-*2*-oleoyl-*sn*-glycero-*3*-phosphocholine (POPC), *1*,*2*-dimyristoyl-*sn*-glycero-*3*-phosphocholine (DMPC), *1*,*2*-dipalmitoyl-*sn*-glycero-*3*-phosphocholine (DPPC), phosphatidylserine (PS) cholesterol (all from Avanti Polar Lipids, Alabaster, Alabama, US). The lipids were stored in chloroform at -20°C.
Experiments were performed at 20°C. At this temperature, POPC is in a fluid phase (l_d), DPPC in a gel phase (s_o). Liquid ordered (l_o) phase state was obtained by adding 30% of cholesterol in DPPC membrane. Coexisting phase states were obtained by adding given amounts of cholesterol in lipids. 20% or 30% of cholesterol in POPC membrane resulted in l_d/l_o phase state and 20% of cholesterol in DPPC membrane resulted in s_o/l_o phase state.

α-lactose monohydrate, dodecylamine, methanol, hypophosphorous acid and dodecylaldehyde used to prepare catanionic surfactants were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA)
12-(*N*-(*7*-nitrobenzo-*2*-oxa-*1*, *3*-diazol-*4*-yl)amino)dodecanoic acid used to synthesize "FluoCat" probe, and Texas Red were obtained from Invitrogen Molecular Probes (Carlsbad, CA, USA).

2. General analytical characterization

^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on Brucker Avance® 300 spectrometer at nominal frequencies of, respectively, 300.18MHz for ^{1}H, 75.48MHz for ^{13}C and 120MHz for ^{31}P.
Mass spectrometry analyses were performed on a PerkinElmer® Sciex API-365, on an electrospray mode.
Infrared spectra were recorded on a PerkinElmer® FT 1760-X spectrometer (0.5% KBr).
Elementary analyses were performed on a PerkinElmer® 2400 Series II Microanalyser.
Spectrofluorimetry measurements were performed on a Spectrofluorimeter PTI (Photon Technology International®) equipped with a EIMAC Xenon lamp of 175W. All the slit widths were set at 2 nm.

3. Surfactant syntheses

The catanionic surfactant 1-N-dodecylammonium-1-deoxylactitol-bis(α-hydroxydodecyl) phosphinate (3) used for the self-assembly of the vesicles was obtained from two amphiphilic precursors: *N*-dodecylamino-*1*-deoxylactitol (1) and *bis*(hydroxydodecyl)phosphinic acid (2).

<u>*N*-dodecylamino-*1*-deoxylactitol (1)</u>

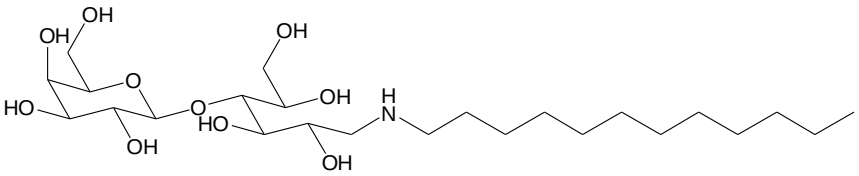

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Fig. S1 Chemical structure of *N*-dodecylamino-1-deoxylactitol (1)

The precursor of the cationic surfactant, N-dodecylamino-1-deoxylactitol (1) (see Figure S1), was synthesized through a reductive amination of the dodecylamine on α-lactose monohydrate, through a two-step reaction allowing (i) the amination of α-lactose monohydrate by dodecylamine, leading to the formation of an amine in equilibrium with the opened-form imine and (ii) the reduction of this imine by H2.
This one-pot reaction was performed in an autoclave. A mixture of α-lactose (8.75mmol, 3.15g) and dodecylamine (14.88mmol, 2.76g) was dissolved into 25mL of methanol, then heated at 50°C for 3 days in the presence of Pd/C (5% mass) under 20 bars of hydrogen. The medium was filtered on celite to remove the palladium, and washed with 200mL ultrapure water/methanol (1:1) at 50°C. Solvents were removed by evaporation and freeze-drying in order to get a white powder. (m=1,62g, R=36%)

- ^{1}H NMR (D_2O, 300MHz): δ (ppm) : 0.81 (m, 3H, CH_3) ; 1.48 (m, 20H, aliphatic CH_2) ; 3.48 to 3.84 (m, 20H, OH, CH and CH_2 of the sugar moiety) ; 4.42 (d, 1H, anomeric H)
- ESI/MS (H_2O): m/z : 512.4 $(M+H)^+$
- IR: ν_{max} (cm^{-1}) : 3435 (N-H st (secondary amine), alcohols), 2924 (C-H st), 2853 (CH st, CH_2 st, CH_3 st), 1638 (N-H st), 1466 (CH_2 δ), 1380 (C-H), 1079 (C-N st (secondary amine)), 720 (N-H δ with aliphatic chain containing more than 4C)
- Elementary analysis: C: 53.90%; H: 9.68%; N: 2.79%, Pd<0.02% (Theory: C: 56.34%; H: 9.65%; N: 2.74%)

<u>Bis(hydroxydodecyl)phosphinic acid (2)</u>

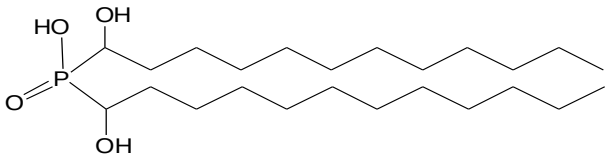

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Fig. S2 Chemical structure of bis(hydroxydodecyl)phosphinic acid (2)

The precursor of the anionic surfactant, the bis-adduct *bis*(hydroxydodecyl)phosphinic acid (2) (see Figure S2), was synthesized according to a standard procedure previously reported in the literature [1]. The addition of hypophosphorous acid to dodecylaldehyde led to the formation of the bis-adduct (2) by an Abramov reaction. (m=2.00g, R=20.5%).

- RMN ^{1}H ($CDCl_3/CD_3OD$, locked on CD_3OD, 55°C, 300MHz): δ (ppm) : 0.85 (t, 6H, CH_3); 1.25 (m, 36H, aliphatic CH_2); 1.61 (m, 4H, CH_2 β); 1.77 (m, 4H, CH_2 α); 3.60 (m, 2H from CHOH)
- RMN ^{13}C ($CDCl_3/CD_3OD$, locked on CD_3OD, 55°C, 300MHz): δ (ppm) : 13.6 (s, CH_3) ; 22.4 to 31.7 (CH_2), between 60 and 70 (CH)
- RMN ^{31}P ($CDCl_3/CD_3OD$, locked on CD_3OD, 55°C, 300MHz): δ (ppm) : 45.43 and 46.52 : due to the phosphorus pseudo-asymetry
- ESI/MS (H_2O): m/z : 433.3 $(M-H)^-$
- IR: ν_{max} (cm^{-1}) : 3313 (C-OH) ; 2918 (C-H) ; 2848 (C-H) ; 2369 (P-OH) ; 1465 (δCH_2) ; 1222 (P=O); 1141 (P=O); 1115 (PO-OH); 1067 (P-OH); 960 (P-OH); 941 (P-OH)
- Elementary analysis: C: 65.67%; H: 9.18% (Theory: C: 66.32%; H: 11.83%)

1-N-dodecylammonium-1-deoxylactitol-bis(α-hydroxydodecyl) phosphinate (3)

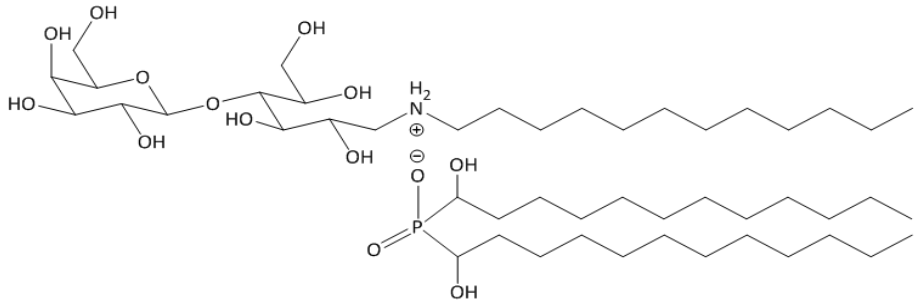

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Fig. S3 Chemical structure of 1-*N*-dodecylammonium-1-deoxylactitol-bis(α-hydroxydodecyl)phosphinate (3)

The tricatenar catanionic surfactant *1-N*-dodecylammonium-*1*-deoxylactitol-*bis*(*α*-hydroxydodecylphosphinate) (3) ‘see Figure S3) was obtained via a spontaneous acid–base reaction in water by addition of the bis(hydroxydodecyl)phosphinic acid (2) to an aqueous solution of N-dodecylamino-1-deoxylactitol (1) (1/1 - mol/mol). The initial heterogeneous suspension turned to a viscous milky solution when the reaction was complete. The reaction was followed by pH measurements until it stabilized. The final product, obtained in quantitative yield, was freeze-fried and stored until use.

- IR: ν_{max} (cm^{-1}) : 3402 (N-H st (secondary amine), O-H st (alcohols)), 2922 (C-H st), 2849 (CH st, CH_2 st, CH_3 st), 2362 (P-OH), 1635 (N-H st), 1469 (CH_2 δ), 1376 (C-H),), 1141 (P=O), 1119 (PO-OH), 1079 (C-N st (secondary amine), C-O st), 1067 (R_2-PO2-), 720 (N-H δ with aliphatic chain containing more than 4C)
- Electrospray, m/z : 946.0 $[M+H]^+$

N-hexadecylammonium-1-deoxylactitol 12-(-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) dodecanoate (FluoCat, (4))

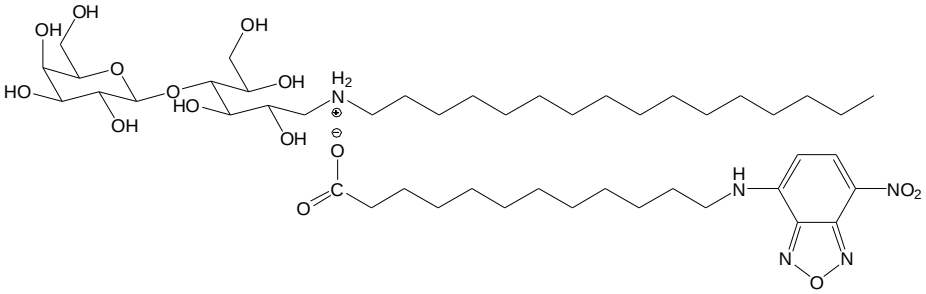

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Fig. S4 Chemical structure of *N*-hexadecylammonium-1-deoxylactitol 12-(-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoate (FluoCat) (4)

In order to label the vesicle bilayers, a fluorescent catanionic surfactant, N-hexadecylammonium-1-deoxylactitol 12-(-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodécanoate (4), so-called FluoCat (λ_{ex} = 488nm) (see Figure S4) , was synthesized according to a standard procedure previously reported in the literature [2].

- IR: ν_{max} cm^{-1}: 3378 (O-H st); 2918 and 2850 (C-H st); 1562 (COO- st as); 1463 (CH_2 δ); (1407 (COO- st as).
- Electrospray, m/z : 946 (cation: 377.4, anion: 568.6)
- ^{1}H NMR (CD3OD, AVANCE 500MHz Bruker, cryoprobe TCI) δ (ppm): 0.93 (t, 3H, CH_3), 1.33 (m, 40H, CH_2), 1.62 ($CH_2CH_2COO^-$), 1.81 ($CH_2CH_2NH_2^+$), 2.20 (CH_2COO^-), 3.34 (CH_2NH, $CH_2NH_2^+$), 3.52 to 3.84 (sugar moiety), 4.15 (NH), 6.38 (NH_2^+), 8.57 ($CHCNO_2$)

4. Catanionic vesicles formation in buffer solution

Non-labeled vesicles

Product (3) spontaneously form catanionic vesicles in aqueous solution when its concentration is above its critical aggregation concentration (CAC = 3.10^{-5} M).
Catanionic vesicles of (3) were formed in a buffer solution allowing the phase contrast visualization of GUVs, composed of 260 mM glucose, 1 mM NaCl and 1 mM Hepes (pH 7.35). The osmolarity matching of the solutions added to the GUVs was checked to avoid osmotic inflation and thus destabilization effects on them.
To improve reproducibility of vesicle formation, freeze-dryed product (3) was put at 1.10^{-4} M in previous buffer solution, stirred and then sonicated (Vibra Cell, Bioblock Scientific® with a titanium probe, pulse rate: 30%, intensity: x3) for 15 min.

FluoCat-labeled vesicles

In order to visualize the interactions between GUVs and catanionic vesicles, the fluorescent catanionic surfactant analog, FluoCat, was co-incorporated in the amphiphilic bilayer of catanionic vesicles (5% - mol/mol).
FluoCat-labeled vesicles were prepared with a mix of freeze-dryed product (3) and FluoCat (4) weighted in molar ratio (95:5), put at 1.10^{-4} M in (3) in previous buffer solution(260 mM glucose, 1 mM NaCl and 1 mM Hepes (pH 7.35).), stirred and then sonicated (Vibra Cell, Bioblock Scientific® with a titanium probe, pulse rate: 30%, intensity: x3) for 15 min.
To check insertion of FluoCat surfactant inside vesicles-bilayers, fluorescent measurements were performed onto vesicles of TriCat/FluoCat (95:5). The excitation wavelength was set at 490 nm and the emitted intensity was collected from 500 to 600 nm. A bathochrome effect resulted from insertion inside bilayers, compared to free FluoCat surfactant in the same medium (See Figure S5).

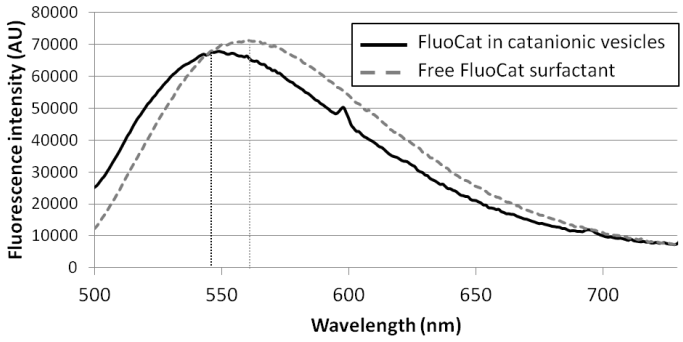

Fig. S5 Fluorescence emission spectra of FluoCat (4) in a free state and in catanionic vesicles composed of Product (3)/FluoCat (95:5) (λ_{ex} = 488nm).

Texas Red labeled vesicles

Texas Red, which is a hydrophilic fluorescent probe (λ_{ex} = 563nm), was encapsulated at 500 µM inside the aqueous core of the vesicles during their self-assembly. On this purpose, Texas Red was dissolved in previous buffer solution (260 mM glucose, 1 mM NaCl and 1 mM Hepes (pH 7.35).)(500µM) before vesicle formation. Vesicles were then formed as previously described, at 1.10^{-4} M in (3).

5. Physicochemical characterization of catanionic vesicles

Non-labeled, FluoCat labeled and Texas Red labeled catanionic vesicles hydrodynamic diameters were evaluated by dynamic light scattering (Zetasizer NanoZS ZEN3600, Malvern Instruments®), to be 200 nm (PI<0.3) in the three cases (See Figure S6 A). Each analysis was performed with a laser wavelength of 633nm, a scattering angle of 173° and at a temperature of 25°C.

Laser-doppler electrophoresis measurements were carried out by Laser Doppler Velocytometry (Zetasizer NanoZS ZEN3600, Malvern Instruments®). From the obtained electrophoretic mobility, the zeta potential (ζ) was calculated using the Smoluchowski equation, to be -30 mV in the three cases (See Figure S6 B).

The formation of the vesicles was observed through Transmission Electron Microscopy (TEM) using a JEOL® JEM 1011 electron microscope, operating at 100kV. Mixtures of catanionic associations in previous buffer solution (10^{-3}M) were applied on copper grids (Formvar®), negatively stained with a 2% (wt/vol) of sodium phosphotungstate (pH 7.5). Vesicles of spherical shapes of about 180nm were observed in the three cases.

Thus physicochemical characteristics of labeled vesicles (with FluoCat or Texas Red) were checked to remain unaffected, as shown in Fig. S6.

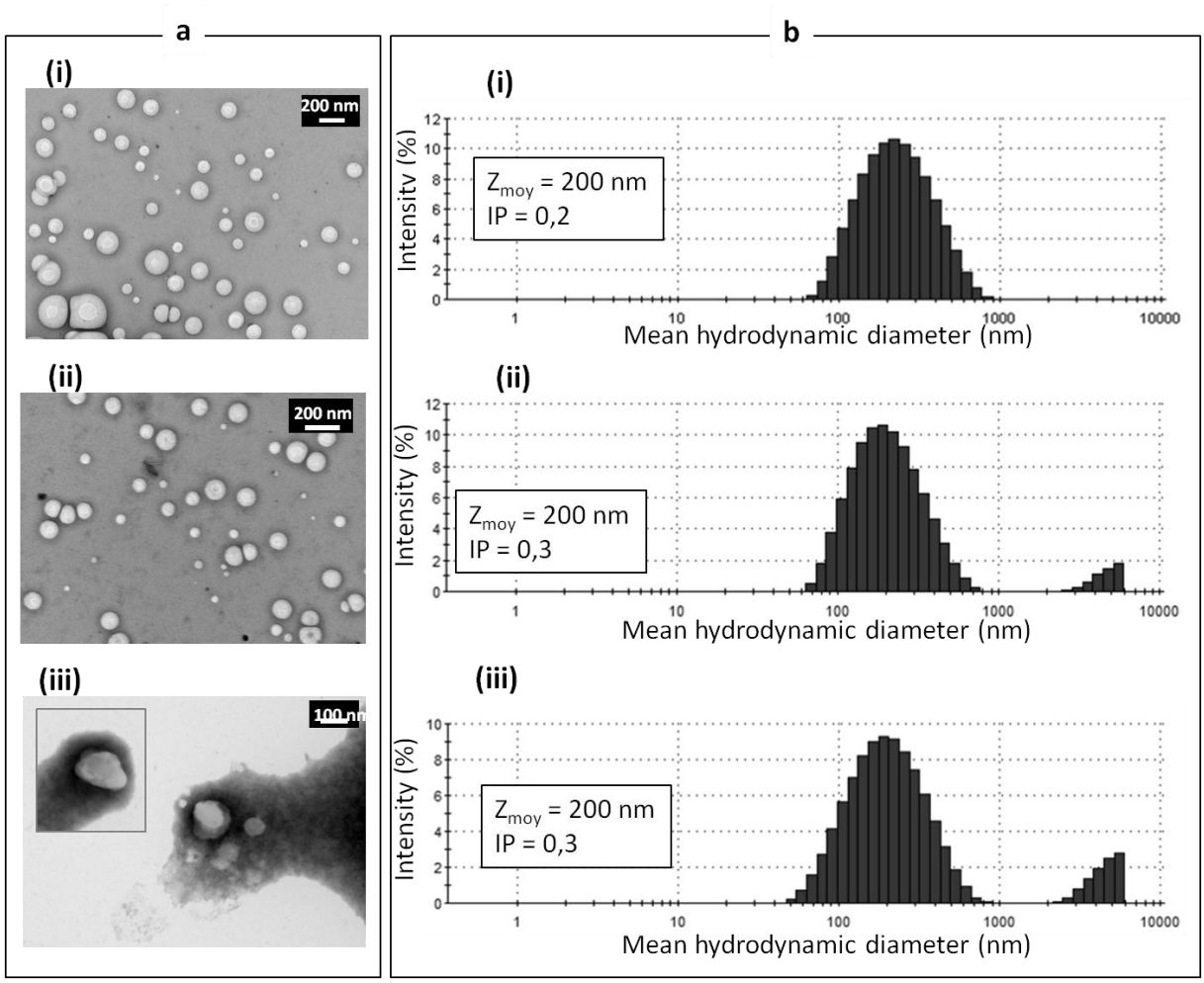

Fig. S6 (a) Transmission electronic microscopy snapshots and (b) dynamic light scattering analyses of (i) non-labeled vesicles, (ii) FluoCat-labeled vesicles and (iii) Texas Red labeled vesicles. All vesicles are formulated at 1.10^{-4} M in (3) in buffer solution.

6. Observation of the interactions between GUVs and catanionic vesicles

Giant unilamellar vesicles composed of Egg-PC, POPC, DOPC, DMPC, DPPC with or without PS and/or cholesterol were produced by the method of electroformation [3]. Their internal buffer is composed of 240 mM sucrose. When present in a buffer, containing 260 mM glucose, 1 mM Hepes and 1mM NaCl (pH 7.35), they can be observed by phase contrast due to the difference of refractive index between the buffer and the internal content.

The observation chamber consisted of a glass slide with a pair of parallel copper tapes spaced of 1 mm, covered by 2 layers of parafilm to create a channel to inject GUVs and catanionic vesicles. The chamber was closed by a coverslip stuck on the parafilm layers. 1.5 µL of the GUVs solution was put inside the chamber and was then diluted with 50 µL of the external buffer. The external buffer was composed of 260 mM glucose, 1 mM NaCl and 1 mM Hepes (pH 7.35). Catanionic vesicles were injected in the chamber just before observation (t=0) at 1.10^{-4} M or 2.10^{-5} M (higher concentrations induce bursting of all types of GUVs; lower concentration doesn't allow the visualization of the interactions). The optical visualization was performed by using an inverted confocal fluorescence Zeiss LSM 510® microscope equipped with a 63x oil objective (for FluoCat experiments) or by using a wide field inverted Leica DM IRB® microscope (PHACO 2 for phase contrast and fluorescence detections) equipped with a 40x objective. Pictures were taken with a digital Quantem

512SC® camera mounted on the microscope and connected to a computer [4]. Sample illumination was achieved with a mercury lamp and a dichroic mirror at an excitation of 488 nm or by phase contrast (Phaco 2) with a halogen lamp.

7. Data processing for content release

Image analysis was performed by using the Metavue software. Two different methods were used to detect aqueous content release from catanionic vesicles to lipid vesicles. The first one is the release of glucose contained by catanionic vesicles inside the GUVs detected by a loss of liposome phase contrast. GUVs were filled with sucrose medium and suspended in a glucose solution to get a good optical contrast. About 300 GUVs were observed after 20 minutes of catanionic vesicle presence and the relative number of GUVs that had lost their contrast was counted. The second one is the release of a hydrophilic probe, Texas Red (λ_{ex} = 563nm), encapsulated at 500 µM by catanionic vesicles inside the GUVs and detected by fluorescence increase in GUVs after 20 minutes of catanionic vesicle presence. Intensity profiles were taken across the GUVs (N = 10) from the wide field images. These profiles present a bell shape illustrative of a homogeneous distribution of Texas Red. The mean intensity of the inside fluorescence of GUVs was measured after correction of the background (due to non-encapsulated Texas Red) to highlight the internal fluorescence of Texas Red. An average on 10 GUVs was done to determine a mean value of fluorescence intensity. This method was used on GUVs mixed with catanionic vesicles loaded with Texas Red and on GUVs in a Texas Red solution, as a control where no fluorescence was detected in the internal volume.

8. Control of the non-insertion of free single catanionic molecules into GUVs membrane

Fluocat was added to the GUV suspension at a low concentration (below its critical aggregation concentration). The putative interaction was observed under the confocal microscope. As shown on Fig S7, no interaction (bilayer fluorescence labeling) was present. The free surfactant under the CAC cannot be spontaneously inserted.

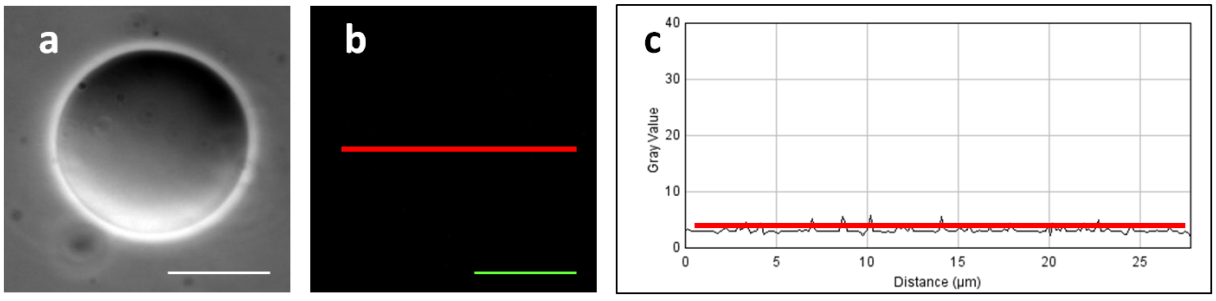

Fig. S7 (a) GUV of DPPC/20% cholesterol observed by phase contrast microscopy, after interaction with free single catanionic molecules. (b) The same GUV after interaction with free single catanionic molecules, observed by fluorescence microscopy. (c) Fluorescence intensity profile measured on GUV diameter (red line). Adding free single Fluocat catanionic molecules doesn't show direct insertion in the membrane of GUVs; as no membrane fluorescence was observed. Scale bar represents 10 µm.

9. Data processing for the determination of membrane fluorescence

Confocal pictures of single GUVs were processed by the software Metavue®. Intensity profiles were taken along a radial line plot of the GUVs. Lipid membrane was detected by two

intensity peaks when an interaction was present (Fig S8) proving the Fluocat transfer in the GUV bilayer.

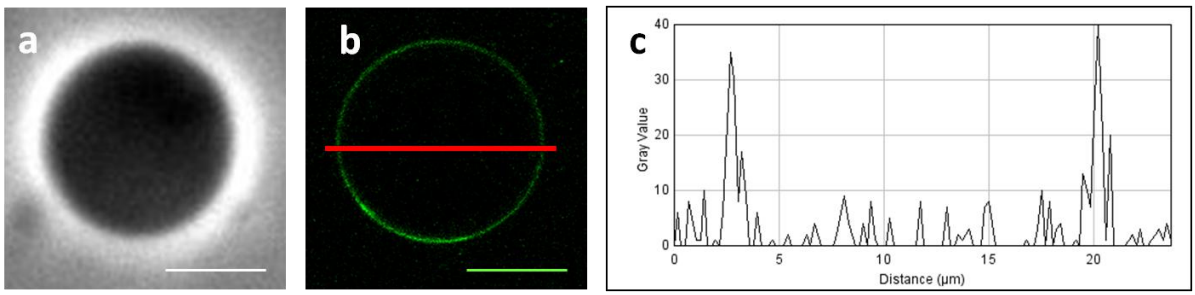

Fig. S8 (a) GUV of DPPC/20% cholesterol observed by phase contrast microscopy, after interaction with FluoCat labeled catanionic vesicles. (b) The same GUV after interaction with FluoCat labeled catanionic vesicles, observed by fluorescence microscopy. (c) Fluorescence intensity profile measured along a GUV diameter (red line). The peaks represent the GUVs membrane that is fluorescent due to the fusion with FluoCat labeled catanionic vesicles. Scale bar represents 10 µm.

Supplementary Videos:

1. A 2min30s video (accelerated) showing the bursting of POPC GUVs (l_d phase state) in the presence of catanionic vesicles at 1.10^{-4} M.

2. A 2min30s video (accelerated) showing that bursting doesn't occur for DPPC/20% cholesterol GUVs (s_o/l_o phase state) in the presence of catanionic vesicles at 1.10^{-4} M.

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[3] M. I. Angelova, D. S. Dimitrov, *Faraday Discussions of the Chemical Society* **1986**, *81*, 303-311.
[4] T. Portet, F. Camps i Febrer, J. M. Escoffre, C. Favard, M. P. Rols, D. S. Dean, *Biophys J* **2009**, *96*, 4109-4121.